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Protein-transitions in and out of the dough matrix in wheat flour mixing

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Abstract:

Sequential protein behavior in the wheat dough matrix under continuous mixing and heating treatment used Mixolab-dough samples from two Australian wheat cultivars, Westonia and Wyalkatchem. High performance liquid chromatography (SE-HPLC) and two-dimensional gel electrophoresis (2-DGE) analysis indicated that 32 min (80 ℃) was a critical time point in forming large protein complexes and loosing extractability of several protein groups like y-type high molecular weight glutenin subunits (HMW-GSs), gamma-gliadins, beta-amylases, serpins, and metabolic proteins with higher mass. Up to 32 min (80 ℃) Westonia showed higher protein extractability compared to Wyalkatchem although it was in the opposite

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direction thereafter. Twenty differentially expressed proteins could be assigned to chromosomes 1D, 3A, 4A, 4B, 4D, 6A, 6B, 7A and 7B. The results expanded the range of proteins associated with changes in the gluten-complex during processing and provided targets for selecting new genetic variants associated with altered quality attributes of the flour.

Key words: Mixolab; SE-HPLC; 2-DGE; wheat dough protein behavior

1. Introduction

Wheat is unique among the cereals with respect to its ability to generate viscoelastic dough, required for a wide diversity of foods (Bushuk, 1998) . A complex mix of proteins and starch are the two broad components in wheat kernel responsible for dough properties that determine the end-use quality of common wheat. Wheat grain proteins have been classified into three major groups: Glutenin, Gliadins, Albumins/Globulins, with each group comprised of suites of individual polypeptides (Juhász, Gell, Békés, & Balázs, 2012).

The roles of different protein fractions on dough processing quality have long demonstrated that gluten (glutenins and gliadin) proteins complexes (P. I. Payne, Nightingale, Krattiger, & Holt, 1987), form polymers with viscoelastic properties. Furthermore, glutenins are divided based on molecular size of the proteins and named as high molecular weight glutenin subunits (HMW-GSs) and Low molecular glutenin subunits (LMW-GSs) (P. I. Payne & Corfield, 1979). Although HMW-GSs only account for 5% to 10% of the total protein, they form the network skeleton of the gluten structure, determining 35-40% of wheat processing quality (P. Payne, Holt, & Law, 1981; Weegles, 1996). Thus the influences of this group of protein on dough quality have been a research focus (Don, Mann, Bekes, & Hamer, 2006; Li, Li, Chen, Kou, Ning, Yuan, et al., 2015; P. I. Payne & Lawrence, 1983). However, LMW-GSs play an important role as well through their contribution to the viscoelasticity and extensibility of dough (Gupta & MacRitchie, 1994; Maucher, Figueroa, Reule, & Peña, 2009).

It is in fact evident that the influence of gluten on dough quality can be more accurately assessed if variation in both HMW-GSs, LMW-GSs, alpha, beta-, gamma- and omega-gliadins were considered as a group in affecting flour-to-dough attributes (Eagles, Hollamby, Gororo, & Eastwood, 2002; Gupta & MacRitchie, 1994; Jin, Zhang, Li, Mu, Fan, Xia, et al., 2013).

The glutenin polymer structure, size distribution, subunit composition and the gliadin/glutenin ratio are major factors in determining dough quality and, hence, the breadmaking potential of wheat flour (D'Ovidio & Masci, 2004; Janssen, Van Vliet, & Vereijken, 1996; Khatkar, Bell, & Schofield, 1995; Uthayakumaran, Gras, Stoddard, & Bekes, 1999). Although it is generally accepted that gluten proteins play an essential role in flour dough rheological properties, non-gluten proteins are clearly important. For example, several endogenous wheat enzymes, proteases (Bleukx, Brijs, Torrekens, Van Leuven, & Delcour, 1998), endoxylanases (Cleemput, Hessing, van Oort, Deconynck, & Delcour, 1997; Cleemput, Van Laere, Hessing, Van Leuven, Torrekens, & Delcour, 1997), enzyme inhibitors, protease inhibitors (Stauffer, 1987) and xylanase inhibitors (Debyser, Derdelinckx, & Delcour, 1997) affect bread-making performance. Avenin-like proteins can influence dough rheological properties by sulfhydryl/disulphide crosslinks with gluten proteins (Ma, Li, Li, Liu, Liu, Li, et al., 2013). It is also reported that polymeric storage globulins are associated with low bread-making performance (MacRitchie, 1987).

Starch as the major component of wheat kernel, it also plays an important role in dough formation. Starch mainly consists of two types of carbohydrate polymers, linear amylose and highly branched structurally complex amylopectin. Starch components generally positioned into the gluten skeleton to form a continuous network of particles, which give rise to viscoelastic behavior (Amemiya & Menjivar, 1992) and consequently influence the final texture and stability of food products after baking (Cuq, Abecassis, & Guilbert, 2003; B.

Lagrain, Wilderjans, Glorieux, & Delcour, 2012; Miyazaki, Van Hung, Maeda, & Morita, 2006).

In this study, Mixolab-dough samples of Westonia and Wyalkatchem, two Australian hard wheat cultivars were collected at seven time points during the dough mixing process. Samples were analyzed by SE-HPLC firstly to observe the polymerization dynamics of different protein fractions. The 2-DGE followed by mass spectrometric protein identification provided further detailed protein characterisation. The aims of this study are to provide an analysis of changes in the dough-matrix during dough formation, and thereafter, in order to define key proteins that can moderate the process and define targets for selecting new genetic variants and associated altered quality attributes of the flour.

2. Material and methods

2.1 Materials

Seeds of two wheat cultivars, Westonia and Wyalkatchem, classified as Australian Premium White were supplied by the Department of Agriculture and Food, Western Australia (DAFWA) and milled into white flour on a Buhler laboratory mill (Brabender Quadrumat Junior mill). The dough mixing was carried out on a Chopin MixoLab, using AACC method 54-6001. Triplicate dough samples were collected from seven time points of mixolab operation: 1.6min or C1 (30 ℃, maximum torque to development time), 3min (30 ℃, close to C1), 17min (32 ℃, initial point of heating), 27min/C2 (56 ℃, dough weakening minimum), 32min/C3 (80 ℃, thermal pasting peak), 38min/C4 (85 ℃, peak of dough temperature), 43min (80 ℃, dough viscosity and temperature returned to the same level of C3). Samples were frozen in liquid nitrogen, freeze-dried and finely ground using a mortar and pestle. Flour samples were used as a control.

2.2 SE-HPLC

To determine glutenin, gliadin and albumin/globulin content, SE-HPLC was carried out (Rakszegi, Bekes, Lang, Tamas, Shewry, & Bedő, 2005) using ten milligram flour or Mixolab-dough power. A sequential protein extraction procedure was followed that included an initial extraction by 0.5% (v/v) SDS-phosphate buffer (pH 6.9), then the remaining insoluble protein fraction was re-suspended in the same buffer by sonication for 15s. After centrifugation, the supernatants were filtered on a 0.45 mm PVDF filter. Analyses were performed on a Phenomenex BIOSEP-SEC 4000 column in an acetonitrile buffer (0.05% (v/v) triflouroacetic acid and 0.05% (v/v) acetonitrile) with a running time of 10 min (2 ml/min flow rate). Proteins were detected by absorption at 214 nm.

Six areas were obtained from soluble and insoluble extracts on the two chromatograms. P1s and P1i indicate the soluble and insoluble glutenin; P2s and P2i indicate soluble and insoluble gliadin; P3s and P3i indicate soluble and insoluble albumin/globulin. The formulas for calculating the content of different protein fractions are following:

Glutenin content (%) = 100* (P1s+P1i)/(P1s+ P2s+ P3s +P1i+ P2i+ P3i)

Gliadin content (%) = 100* (P2s+P2i)/(P1s+ P2s+ P3s +P1i+ P2i+ P3i)

Albumin/globulin content (%) = 100* (P2s+P2i)/(P1s+ P2s+ P3s +P1i+ P2i+ P3i)

UPP content (%) = 100* P1i/(P1i+P1s)

UG content (%) = 100* P2i/(P2i+P2s)

UAlb/Glob content (%) = 100* P3i/(P3i+P3s)

2.3 2-DGE

Protein fractions of flour or Mixolab-dough samples were analysed by 2-DGE (Islam, Ma, Yan, Gao, & Appels, 2011). Non-gluten proteins (abbumin and globulin) were extracted with 0.5 M NaCl (Ph 7.0) at 100mg/mL under non-reducing and non-denaturing conditions. The suspension was stirred at 4 ℃ for 5 h, and insoluble materials removed by centrifugation at

12000g for 20min. Non-gluten proteins in the supernatant were precipitated by incubation with 4 volumes of ice-cold acetone at -20 ℃ for 14 h, and recovered by centrifugation. Finally, the protein pellet was washed with 10% ethanol and then with acetone containing β-mercaptoethanol (0.07%) to remove remaining salts.

For gluten protein extraction, five commonly used buffers, possessing different levels of strength and biochemical properties were used. Gluten proteins were extracted from flour and two Mixolab-dough samples (C1/1.6 min and C3/32 min) using four different buffers at 100mg/mL. Buffer SD (0.3% SDS + 15 mM DTT) with medium dissociation was then used to extract gluten proteins from the residue left after extracting non-gluten proteins. Buffer UCD (8 M Urea + 4% CHAPS + 60 mM DTT) with strong dissociation was selected for the separation of all the gluten proteins. Ten milligram of the recovered non-gluten or gluten protein pellet was dissolved in rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, and 2% IPG buffer for 5 h at room temperature. Protein concentration was determined by using an RC DC protein assay kit (Bio-Rad, Hercules, CA) and a Lambda 25 UV-vis spectrometer (PerkinElmer). For each sample, 1100 μg of protein was loaded onto IPG strips (Bio-Rad). Isoelectric focusing (Wieser & Kieffer) was conducted on 17 cm IPG strips with pH 3-10. The strips were rehydrated with buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, and 2% IPG buffer) containing 1100 μg of protein for 12 h. Strips were focused at 60000 Vh, with a maximum of 10000 V, at 20 ℃ using a Protein IEF cell (Bio-Rad). Before running SDS-PAGE, the strips were equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.002% bromophenol blue containing 65 mM DTT for 15 min and for another 10 min by substituting DTT with 135 mM iodoacetamide in the same buffer. Protein separation was carried out on 12% acrylamide/bis (37.5:1) gels, using a Protein II Xi cell (Bio-Rad). The running buffer consisted of 2.5 mM Tris-Base, 19.2 mM glycine, and 0.01% SDS. The gels were stained in Coomassie Brilliant

Blue (CBB) solution. Protein standards (Bio-Rad) were used to estimate the molecular size of the proteins. To minimize experimental variability, all samples were run three times with individual extraction and IEF. The gels were scanned by a 2-D Proteomic Imaging System, "Image lab 5.0" (Bio-Rad). The digital gel maps of different samples were analyzed and compared using PD Quest software (Bio-Rad).

2.4 Protein identification by MS/MS

Protein spots with different expression patterns were manually excised from gels and analysed further by mass spectrometric peptide sequencing. The spots were analysed by Proteomics International Ltd. Pty, Perth, Australia. Protein samples were trypsin digested and the resulting peptides were extracted according to standard techniques. Tryptic peptides were loaded onto a C18 PepMap100, 3 µl (LC Packings) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v), using an Ultimate 3000 nano HPLC system. The HPLC system was coupled to a 4000Q TRAP mass spectrometer (Applied Biosystems). Spectra were analysed to identify the proteins of interest using Mascot sequence matching software (Matrix Science) with taxonomy set to Viridiplantae (Green Plants). All searches used the Ludwig NR. The software was set to allow 1 missed cleavage, a mass tolerance of ±1.2 Da for peptides and ± 0.6 for fragment ions. The peptide charges were set at 1+, 2+ and 3+, and the significance threshold at $P < 0.05$. Generally, a match was accepted where two or more peptides from the same protein were present in a protein entry in the Viridiplantae database.

3. Results

3.1 Dough matrix in the MixoLab process

3.1.1 Overall profiles of Westonia and Wyalkatchem

The data in Fig.1 shows that the two varieties Westonia and Wyalkatchem were very similar

in mixing properties using the Mixolab. The major differences between the two varieties were between the time points 32min and 43min of the mixing curve. At this stage of the mixing curve the starch had gelatinized and the temperature of the mixing was 80-85℃. In this part of the mixing curve the behavior of Wyalkatchem was typical of udon noodle wheat varieties (Cato & Mills, 2008), whereas Westonia showed a profile which was clearly distinct from Wyalkatchem in that the post 32 min drop in torque was much less. SE-HPLC results showed that 27 min (56 ℃) is a critical time point for the Mixolab assay, with overall protein extractability decreasing after this time point (Fig.2). However, the decreasing pattern was different across the protein groups and between the cultivars. Generally, cultivar Wyalkatchem showed a greater decrease in protein extractability compared to Westonia. This difference between the cultivars was very marked in flour per se and the earliest stage of dough mixing (1.6 mins). Thereafter the properties of the flour from the two varieties were more difficult to differentiate.

3.1.2 Changes in glutenin proteins

A large decrease in glutenin proteins' extractability was observed between 0 min and 43 min (Fig.2) in both cultivars. The decrease in Westonia was 66.4%, comparing with 80.8% decrease in Wyalkatchem. As is shown in Fig. 3, the relative amount of glutenin proteins was not changed significantly before 27 min. However, thereafter the percentage of glutenin polymers decreased by 20.7% and 22.4% in Westonia and Wyalkatchem, respectively. Within the glutenin proteins, the ratio of unextractable proportions (soluble only after sonication) of gluten polymers (UPP%), increased until C4/38min (85 ℃). Pattern of UPP% in Westonia and Wyalkatchem was quite different as in Westonia there was a sharp increase between 27 mins and 38 mins. The behavior of glutenin proteins was further indicated by 2-DGE analysis (Supplementary Fig.1, Supplementary Fig.2 and Supplementary Fig.3). HMW-GSs behaved differently based on their mass weight. Most HMW-GSs with higher mass (x-type) lost their

extractability in SD after C3/32 min (80 ℃) whereas the HMW-GSs with lower mass (y-type) remained extractable. The LMW-GSs readily extracted by SD (Fig. 4 H) or even mild extraction buffer such as 0.5 M NaCl (pH 7.0) (Fig.4, C) after C3 (32 min, 80 ℃).

3.1.3 Changes in gliadins

The extractability of gliadins, was decreased on a smaller scale (Fig 2) compared to glutenin proteins. The decrease of gliadins between 0 min and 43 mins in Westonia was 19.2% compared to 38.3% (Fig 2) in the case of Wyalkatchem. The relative amount of gliadins increased 13.5% and 18% respectively in Westonia and Wyalkatchem. The ratio of unextractable proportions (soluble after sonication only) of gliadins (UG %) increased and reached their peak at C4/38min (85 ℃) (Fig. 3). Results of 2-DGE showed that most of the gamma-gliadins lost their extractablity after C3/32 min (80 ℃) whereas alpha/beta-gliadins, and avenin-like proteins remained extractable (Supplementary Fig.1, Supplementary Fig.2 and Fig.4 G, I).

3.1.4 Changes in albumins and globulins

As shown in Fig 2, small scale decreases of extractability of albumins and globulins were observed. While in the case of Westonia, the decrease of albumins/globulins between 0 min and 43 mins was 16.7% (Fig 2) compared to 40.4% (Fig. 2) in the case of Wyalkatchem, the *relative* amount of albumins/globulins only increased 6% and 6.6% respectively. Furthermore the ratio of unextractable proportions (soluble after sonication only) of albumins/globulins (UAlb/Glob %) increased up to C4/38min (85 ℃) (Fig. 3). Protein profiles from 2-DGE showed that albumins/globulins were readily extractable by 0.5 M NaCl (pH 7.0) until C3 (32 min, 80 ℃) and thereafter the extractability of these proteins with higher molecular weight (globulins and metabolic enzymes with molecular weight between 30kDa and 90kDa) dropped significantly (Fig. 4, A), possibly due to interactions with gluten proteins or pasting

starch. In contrast, the albumins or globulins with lower mass such as the majority of alpha-amylase inhibitors and some other proteins with molecular weight between 15kDa and 30kDa, were extractable at every time point (Fig. 4, B).

No differences in extractability of gluten proteins was observed between the different stages of dough mixing and heating when UCD buffer was used for extraction (Supplementary Fig.3), and only a decrease was detected after the C3/32min (80℃) time point, which indicated that the unextractable proportion of gluten proteins by SD were not degraded during the mixing and heating process.

The combined protein behaviours in the three extraction buffers indicated that the C3/32min (80 ℃) was a critical time point in dough mixing in terms of protein extractability. The number of proteins with comparatively higher molecular weight such as beta-amylase, serpin, HMW-GS Dx5 and Dx2 and gamma-gliadins lost their extractability after C3 in both the cultivars. However, alpha/beta-gliadins, some LMW-GSs, HMW-GSs with lower mass, 27K protein, superoxide dismutase and most of the alpha-amylase inhibitors could be extracted from each time point.

Although the buffer 0.5 M NaCl and SD or UCD were particularly used to extract albumins/globulins and gluten proteins respectively, protein fractions overlapping those extracted with the other buffers (Supplementary Table 1) were also observed. For example, beta-amylase, serpin, superoxide dismutase and alpha-amylase inhibitors were extracted by SD/UCD and, interestingly, gluten proteins, such as alpha/beta- and gamma-gliadins, low-molecular-weight glutenin subunits, and avenin-like b proteins were extracted by 0.5 M NaCl (pH 7.0) as well.

3.2 Identification of specific proteins within the dough matrix

Mixolab profiles of Westonia and Wyalkatchem flour samples demonstrated significant

differences between the dough protein matrix of these two cultivars at C4/38min (85 ℃) time point. The protein fractions quantified by SE-HPLC and protein expression characterization by 2-DGE from different stages of dough preparation confirmed the varietal differences. Particularly, significant differences in expression of a number of proteins between the cultivars have been observed after 27 minutes of mixing even though the overall behavior of protein groups was similar across the cultivars.

3.2.1 Differential proteins as extracted by 0.5 M NaCl (pH 7.0)

At C2/ 27min (56 ℃), more proteins were extracted from Westonia than Wyalkatchem (Fig. 5, A, B and C). Glyceraldehyde-3-phosphate dehydrogenase, Malate dehydrogenase, globulin-3A, and beta-amylase were extracted from Westonia (gel area A) but not from Wyalkatchem. Likewise, xylanase inhibitor protein, Class II chitinase and GluB3-6 (gel area B) were only extracted from Westonia. The average amounts of these differentially expressed proteins were listed in Supplementary Table 2. On the other hand, the quantities of the soluble gluten proteins, alpha/beta-gliadins and globulin-3 extracted were higher in Wyalkatchem than Westonia (Fig.5 C) at C4/ 38 min (85 ℃) time point. The abundant differential behaviouors of these proteins between the two cultivars are presented in Supplementary Table 2.

3.2.2 Differential proteins as extracted by SD

Significant differences between Westonia and Wyalkatchem dough proteins' extractability (Supplementary Fig.2) after 27 min were identified with buffer SD. At C2/27min (56 ℃), beta-amylase, alpha/beta-gliadins, avenin-like b proteins, HMW-GSs with low mass, LMW-GSs and some metabolic proteins from Westonia showed higher solubility than Wyalkatchem (Fig. 6 and Supplementary Table. 3). The increasing temperature and over-mixing, was not found to change the relative extractability of proteins from Westonia (remained higher) and Wyalkatchem at C3/32min (80 ℃). As shown in Fig. 6 E, more proteins, including most of

the gamma-gliadin, avenin-like b protein, globulin-1 and some metabolic proteins, were isolated from the dough matrix of Westonia. In contrast, only globulin-3 and some LMW-GSs reflected higher extractability in Wyalkatchem (Fig. 6 E, Supplementary Table. 3). The differentially extracted proteins between Westonia and Wyalkatchem at C3/32min (80 ℃) are listed in Supplementary Table 3.

As mentioned above, C3/32min (80 ℃) is evidently a critical time point of dough mixing and heating. Protein extractability changed after this time point, showing different patterns between the two cultivars. When the dough temperature up to 85 ℃ at C4/38 min, the specific proteins (additional gamma-gliadins, globulin-1, avenin-like b proteins, peroxidase, and enolase-like proteins) only extracted from Westonia at C3/32min (80 ℃) lost their extractability, while more globulin-3, globulin-1 avenin-like proteins and LMW-GSs (the unmatched spots 146, 148, 149 were predicted to be LMW-GSs according to their mass and PI value) were isolated from the dough matrix of Wyalkatchem (Supplementary Table. 3).

Compared to C4/38 min (85 ℃), the abundance of extractable proteins at 43min (80 ℃) time point increased in both the cultivars, yet similar protein profiles were observed at these two time points. Significant increases of globulins, gliadins and LMW-GSs however were observed in Wyalkatchem. Moreover, beta-amylase and x-type HMW-GSs, which were unextractable at C3 and C4, were separated from dough of Wyalkatchem at this time point (Supplementary Table. 3).

4. Discussion

4.1 Assembly of the gluten complex

The de novo assembly of the large gluten complexes in dough formed from wheat flour is a process requiring mechanical energy input and water addition, as the proteins unfold and form a continuous macromolecular viscoelastic network comprised of disulfide bonds, the

arrangements of hydrogen bonds, hydrophobic interactions and entanglements (Mann, Schiedt, Baumann, Conde-Petit, & Vilgis, 2013; Singh & MacRitchie, 2001). The documentation of the extractability of protein classes at different stages of dough formation has been widely explored (Weegels, De Groot, Verhoek, & Hamer, 1994; Weegels, Verhoek, De Groot, & Hamer, 1994) and in the present paper we have followed this experimental approach to define the polymerization of specific proteins within the gluten complex as it is formed in the MixoLab process.

Formation of the gluten complex occurs in the presence of an excess starch and as the heat treatment of the dough takes place the starch granules' hydrophobic surfaces (Seguchi, 1984) and physical properties change, and affect the characteristics of the gluten complex (Mann, Schiedt, Baumann, Conde-Petit, & Vilgis, 2013). Denaturants and reductants are commonly used for the isolation of different protein fractions to observe their responses during dough preparation. SDS and Urea, as denaturants, can change the quaternary and tertiary structure of proteins and expose their disulphide bonds (Weegels, De Groot, Verhoek, & Hamer, 1994), while the reductants, such as DTT and 2-ME, or sonication can be applied to break down these exposed bonds. In this study, proteins were separated by traditional sequential extraction procedure for SE-HPLC analysis; soluble-proteins were extracted by 0.5% (v/v) SDS-phosphate buffer (pH 6.9), and then the remaining insoluble protein fraction was re-suspended in the same buffer by sonication for 15s. However, the solubility of all the protein fractions, especially glutenin, decreased after C3/32 min (80 ℃), indicating not all the disulfide bonds can be broken by sonication after over mixing and heating. As for the 2-DGE analysis, 0.5 M NaCl (pH 7.0) was used for extracting the albumins and globulins, while two buffers, SD (comprising 0.3% SDS and 15 mM DTT) and UCD (8 M Urea, 4% CHAPS, 60 mM DTT), with medium and strong dissociation respectively were used to extract gluten proteins. Our data showed that there were different energy thresholds for breakage of

chemical bonds in dough matrix, especially for disulfide bonds. Buffer SD, with low concentration of SDS and DTT, broke the more accessible disulfide bonds, while the stronger buffer UCD was required to isolate all the proteins not extracted by buffer SD, consistent with the fact that disulfide bonds are mainly responsible for protein insolubility, and that most of the disulphide bonds would be reduced upon buffer with strong dissociation.

4.2 Bonding strength of protein groups in dough matrix

Binding energy of different proteins in dough matrix can be roughly ranked according to their solubility in the three extraction buffers (0.5 M NaCl, SD and UCD) under mixing and thermal treatment. Non-gluten proteins with low molecular weight, viz. 27K protein, superoxide dismutase and most of the alpha amylase inhibitors, can be extracted from dough at every stage during dough preparation by all the three buffers, indicating they distributed in the dough matrix as monomeric proteins and maintain free even after over mixing and heating. Beta amylase, serpin glyceraldehyde-3-phosphate dehydrogenase and some globulins with higher mass lost their extractability by 0.5 M NaCl (pH 7.0) after 27 min, but remained extractable by SD or UCD. It is proposed that these proteins were aggregated into the dough matrix by increased hydrophobicity or linked with gluten proteins through disulphide-sulfhydryl changes after mixing and thermal treatment (Bert Lagrain, Thewissen, Brijs, & Delcour, 2008; Mann, Schiedt, Baumann, Conde-Petit, & Vilgis, 2014).

In contrast, more energy was required to separate the gluten proteins from dough matrix. As shown in Fig.5 C after 32 min, some alpha/beta-gliadins and a small proportion of LMW-GSs were isolated from dough matrix by 0.5 M NaCl (pH 7.0), demonstrating their continuous weakening linkage with the gluten polymers after heating. The avenin-like b proteins, gamma-gliadins and LMW-GSs were extracted with SD, indicating relatively stronger bond. This result also showed that gamma-gliadins were more affected by heating than alpha/beta gliadins, which is consistent with the study by Lagrain et al (2005). HMW-GSs (except

1Dy12.3), as the backbone of dough matrix, lost their extractability in SD after 27 min although they were still extracted by UCD. This phenomenon can be explained as follows: heat treatment induced polymerization of gluten molecules stabilized by disulphide interchanges (Amemiya & Menjivar, 1992) and hydrophobic interactions (Mann, Schiedt, Baumann, Conde-Petit, & Vilgis, 2013). It is possible that these conformational changes in the gluten polymers hinder the more extensive formation of disulphide bonds between other HMW-GSs. For example, the relatively low mass subunits such as 1Dy12.3 were more readily extracted from the developed gluten complex than the respective x-type HMW-GS consistent with previous findings that contributions of the x-type HMW-GSs to dough properties were more important than that of the y-type (lower molecular weight) (Wieser & Kieffer, 2001). We speculate that disulphide bonds formed by x-type HMW-GSs were wrapped in the core of the aggregate and only reduced by buffer with strong dissociation (UCD).

4.3 Protein behaviours in SE-HPLC and 2-DGE

The SE-HPLC analytical approach was used to identify the proportionate changes of different protein groups, such as albumins/globulins, gliadins and glutenins. The 2-DGE analysis was carried out for a high resolution characterization of the specific protein expression through the dough development process. This also allowed us to identify the proteins through mass spectrometric peptide sequencing and to annotate proteins of interest to the wheat genome sequence. The overall protein behaviours identified by SE-HPLC and 2-DGE were very similar. For example, C3/32 min (80 ℃) has been identified as a critical point of dough preparation in both methods since the extractability of total protein from the dough matrix decreased dramatically largely due to the loss of extractability of HMW-GSs, gamma-gliadin, beta amylase, serpin, and metabolic proteins with higher mass. However, 2-DGE demonstrated the potential to characterize the dough protein changes in depth that were not

achieved by SE-HPLC in all the cases. For example in SE-HPLC there was no remarkable difference between the cultivars at C3/32 min (80 ℃) whereas the 2-DGE identified significant differences in the presence of number of proteins like alpha/beta-gliadins, LMW-GSs, globulin-3 and globulin-1 (Fig. 5 C and Fig. 6 F).

4.4 Dough protein behaviour as influenced by genetics

The analysis carried out provides a new view of the difference between the Westonia and Wyalkatchem in the Mixolab curve at C4/38 min (85 ℃) (Fig. 1), where a small drop in torque of about 0.2 N/m, in Westonia's curve and rebounded 3 min later. Wyalchatchem, in contrast showed a sharp drop in torque gap about 0.5 N/m, which lasted till 43 min (80 ℃). Based on the results from the extractability and dough rheological properties, we can visualize dough as a continuous gluten network, into which starch granules are embedded as filler particles and protein conformation can change either through disulfide-sulfhydryl interchange or hydrophobic interactions. At C3/32 min, a large aggregate formed by either disulphide-sulphydryl interchange or hydrophobic interactions, combines with the heat treatment triggering starch swelling and altered starch surface hydrophobicity as a result of denaturation of starch-associated proteins (Mann, Schiedt, Baumann, Conde-Petit, & Vilgis, 2013). All the changes leading to increased torque value are accompanied with differential protein polymerization/depolymerizations within the dough matrix in Westonia and Wyalkatchem, *viz.* additional gamma-gliadin, globulin-1, avenin-like b, peroxidase, and enolase-like proteins were extracted from Westonia, while more globulin-3 and low-molecular proteins were separated from Wyalkatchem. We speculate that the different drops in the Mixolab curve at C4 (Fig 1) were associated with the different mixtures of protein polymerisation/depolymerisation in the two cultivars. We deduce the dough torque of Westonia rebounded within short time due to the additional proteins, gamma-gliadin, globulin-1, peroxidase and enolase-like proteins, that were extracted from Westonia's dough

at C3/32 min (80 ℃) being repolymerised into the dough matrix at C4/38 min (85 ℃). Compared to Westonia, more proteins, alpha/beta-gliadin, globulin-3, globulin-1 and LMW-GSs, were depolymerised from the dough of Wyalkatchem at C4/38 min (85 ℃) and no protein was repolymerised into dough matrix, leading to the sharp drop on its Mixolab curve until 43 minutes when the dough temperature returned to 80℃.

Twenty proteins that differentially extracted in the comparison of Westonia and Wyalkatchem could be allocated to addresses within the wheat genome, single entities could be assigned to chromosomes 1D, 3A, 4D, 7A and 7B, two entities to chromosomes 4B and 6B, three entities to chromosome 3A and four entities to chromosomes 4A and 6A. These assignments expanded the range of proteins traditionally considered to be associated with changes in the gluten-complex during processing. This study has provided new detail for the hierarchical structures of proteins within the dough complex. The identification of specific proteins polymerising and depolymerising from the dough matrix at different stages of the dough processing provides a basis for designing novel interventions using particular proteins as carriers for molecules such as alfa-amylase or protease inhibitors to alter the dough complex. In addition the proteins provide targets for selecting new genetic variants that may be associated with altered quality attributes of the flour.

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Supporting information

Matching of the mass spectrometric peptide sequences to identify the common proteins.

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Figure Legends

Fig. 1 Mixolab Curve showing the dough sampling points: C1/1.6min (30 ℃), 3min (30 ℃), 17min (32 ℃), C2/27min (56 ℃), C3/32min (80 ℃), C4/38min (85 ℃), 43min (80 ℃). The Y axis on the left hand side monitors the temperature changes within the dough during mixing (green line indicated) and the Y axis on the right-hand side is the torque required to maintain the dough mixing action (standard units of torque, Newton/meter, Nm).

Fig. 2 Amount fluctuation of different protein fractions during Mixolab dough preparation as identified by SE-HPLC. Y-axis indicates the relative abundance of the extractable proteins with Westonia flour as a control (abundance is 1.0). The relative amount of the respective protein group was calculated based on the total peak area of proteins within the group as determined by SEHPLC system.

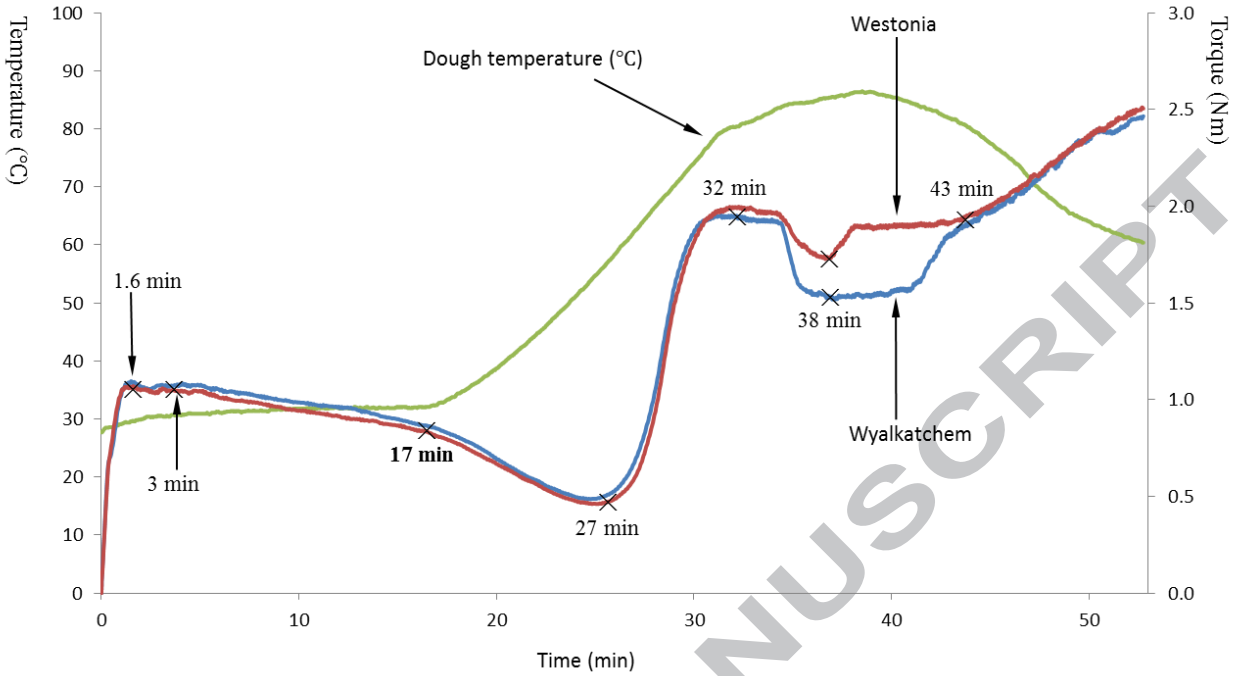
Fig. 3 The relative amounts of different protein fractions extracted from Mixolab dough as identified by SE-HPLC. Y-axis indicates content of different protein fractions in the dough extracts.
After 27min (heating) the extractability of the all the protein fractions decreased (see Fig 2), due to the protein heating, starch pasting and the interaction between the two components, which explains the increase of unextractable proteins. The decrease of Glu% was caused by the large decrease of glutenin proteins (see Fig 2). However, the ratio of the other two protein fractions increased due to the large relative decrease of glutenin proteins even though their absolute amount decreased (see Fig 2).

Fig. 4 Abundance variation of proteins soluble in 0.5 M NaCl (A-C) and buffer SD (D-J) as identified by 2-DGE. Y-axis indicates the dough protein abundance ratio where the extractable protein of Westonia flour or Wyalkatchem flour (proteins only extracted from Wyalkatchem) was set as control. The Spot number indicated their identification by mass spectrometry as listed in the Table. 1 of the supplementary document.

Fig. 5 Comparison of specific regions in the protein profiles of C2/27min (56 ℃) and C4/38 min (85℃), demonstrating the differentially expressed proteins between Westonia and Wyalkatchem. The number on the spots indicated their identification by mass spectrometry as listed in the Table. 2 of the supplementary document. The plates A, B and C refer the regions showed in Supplementary Fig. 1.

Fig. 6. Comparison of specific regions on the protein profiles of C2/27min (56 ℃), C3/32min (80 ℃), C4/38min (85 ℃) and 43min (80 ℃) demonstrating differentially expressed proteins between Westonia and Wyalkatchem. The number on the spots indicated their identification

by mass spectrometry as listed in the Table. 3 of the supplementary document. The plates D, E, F and G indicate the regions showed in Supplementary Fig. 2.



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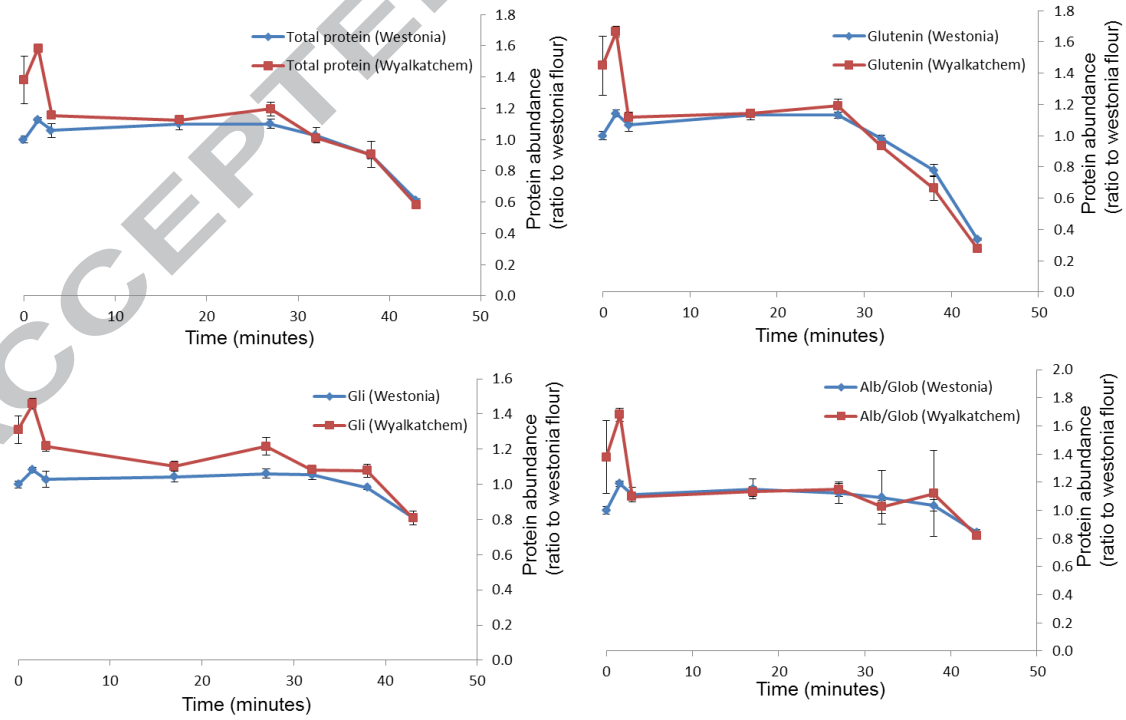

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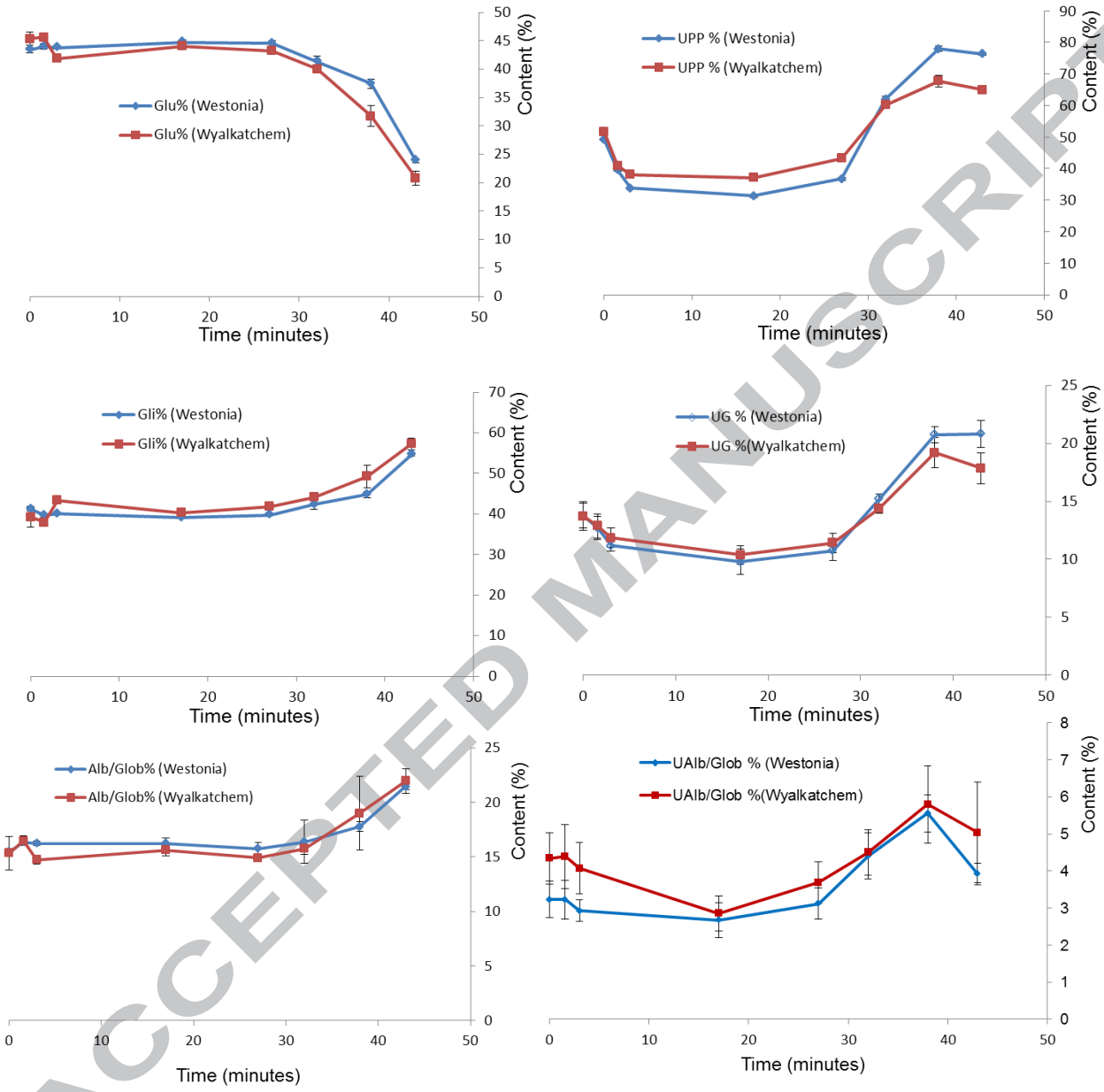

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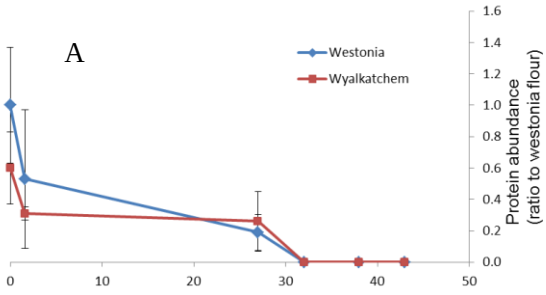
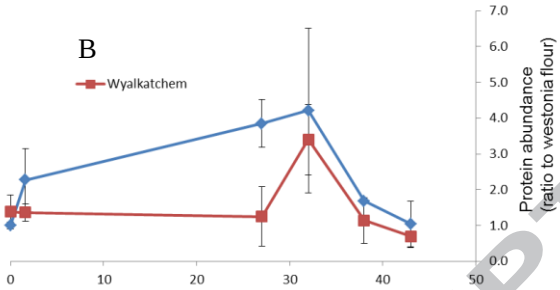
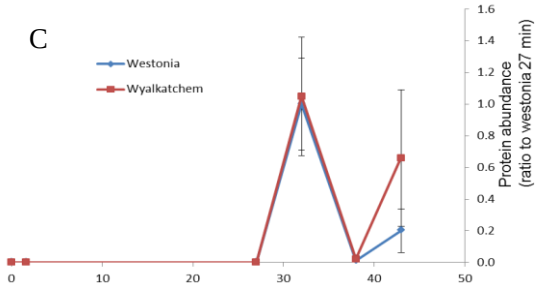


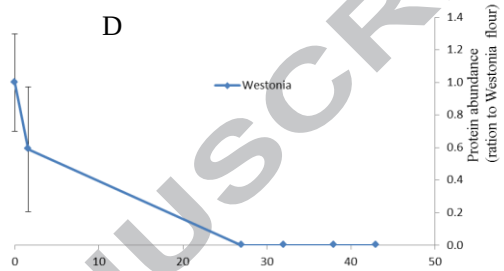
Beta-amylase (Spot 4)



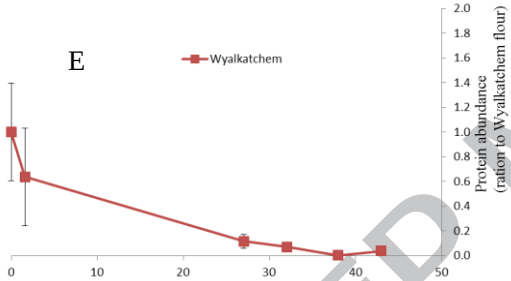
Dimeric alpha-amylase inhibitor (Spot 36)



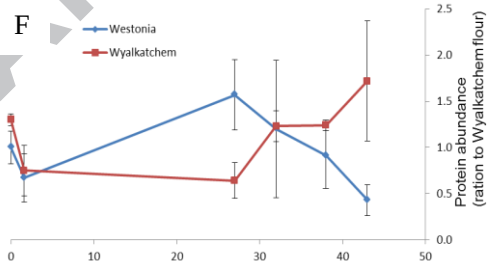
Alpha-gliadin (Spot 76)



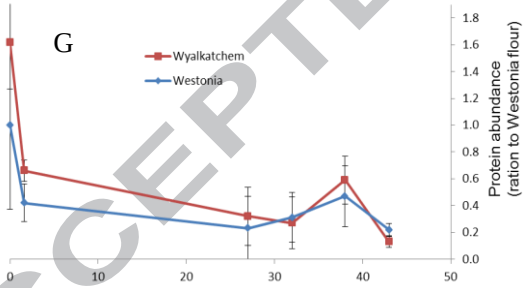
HMW-GS 1Dx2 (Spot 63)



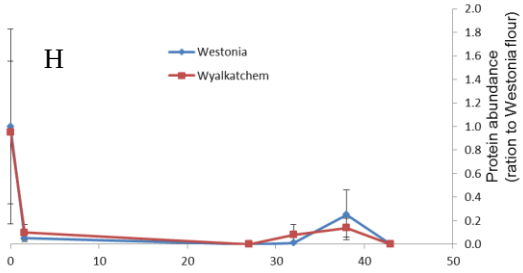
HMW-GS 1Dx5 (Spot 60)



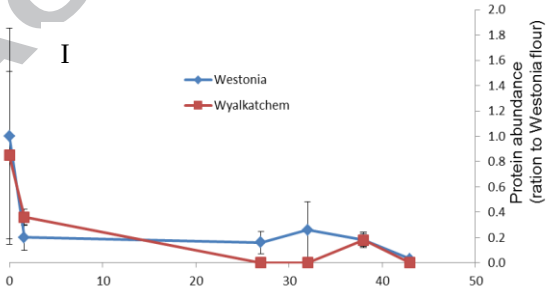
HMW-GS 1Dy12.3 (Spot 122)



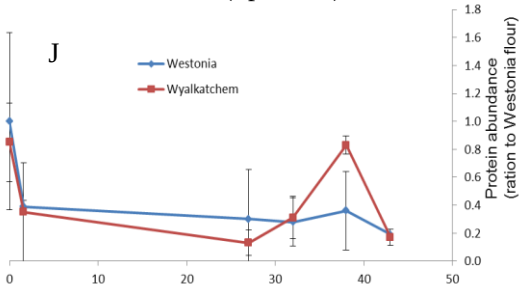
Alpha/beta-gliadin (Spot 83)



GluB3-6 (Spot 145)



Avenin-like b7 (Spot 113)



Globulin-1 S (Spot 77)

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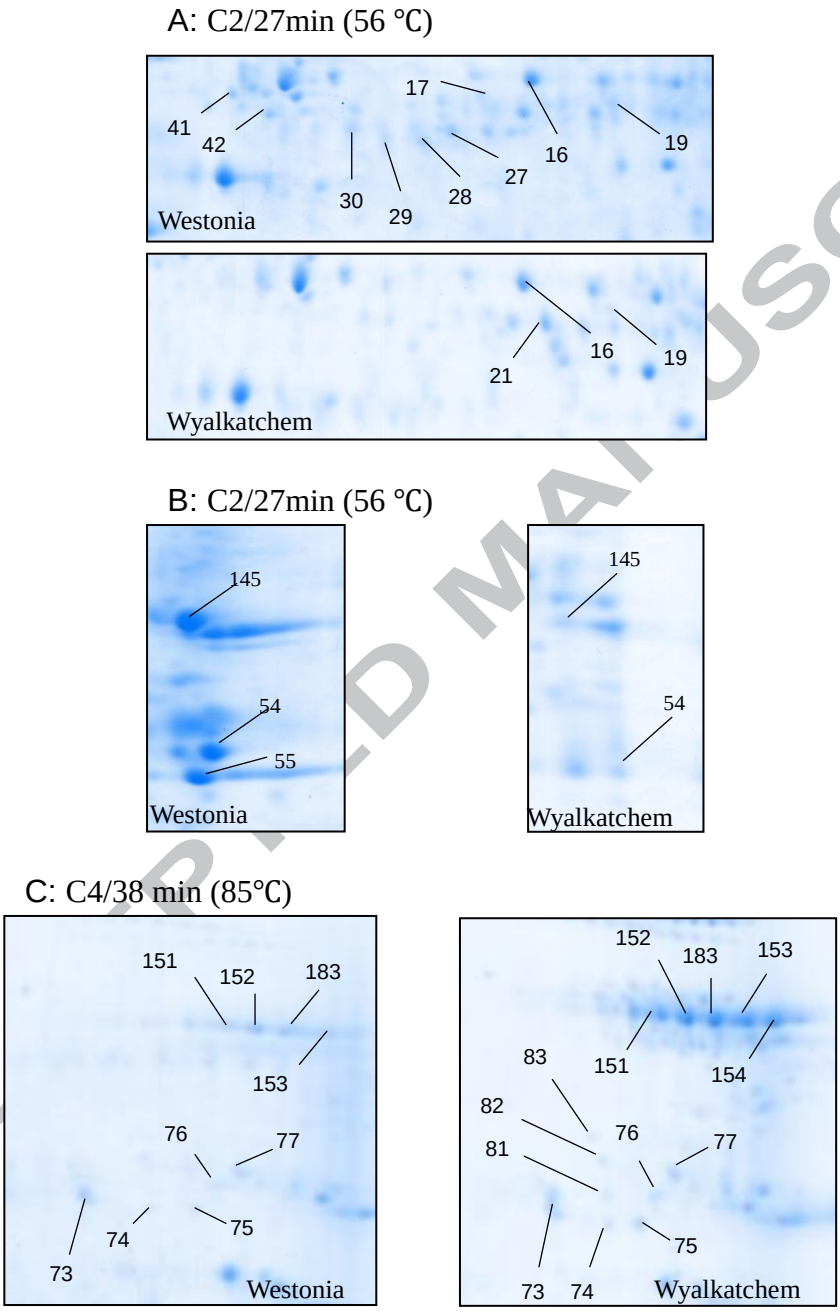

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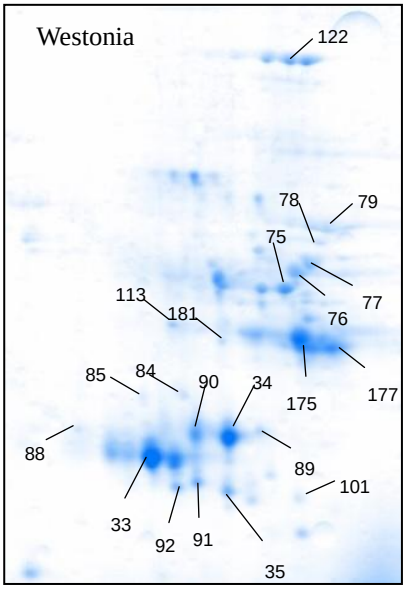
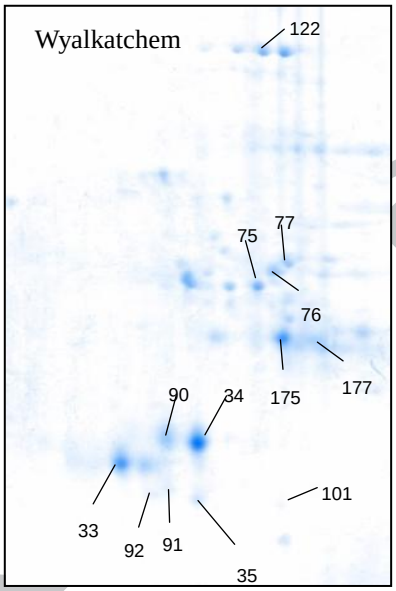
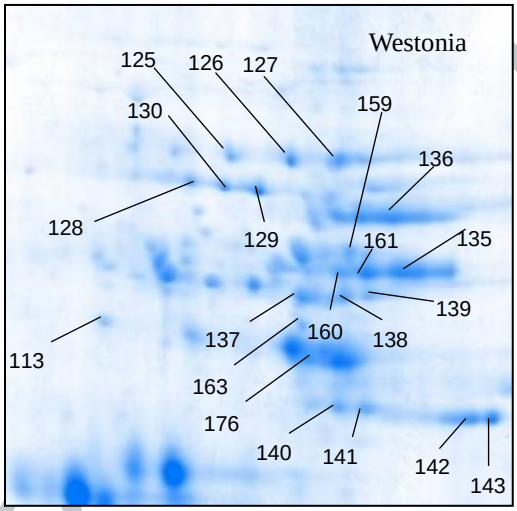
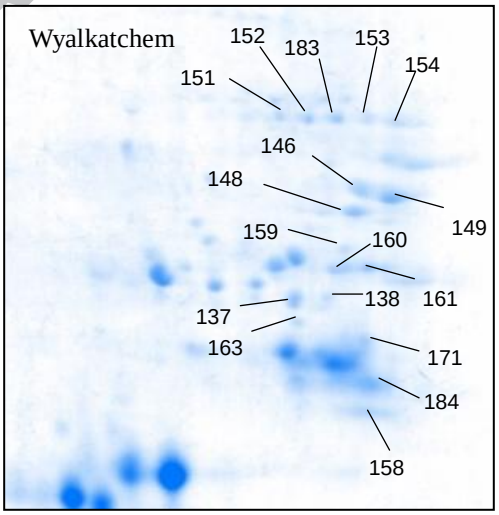
D: C2/27min (56 ℃)



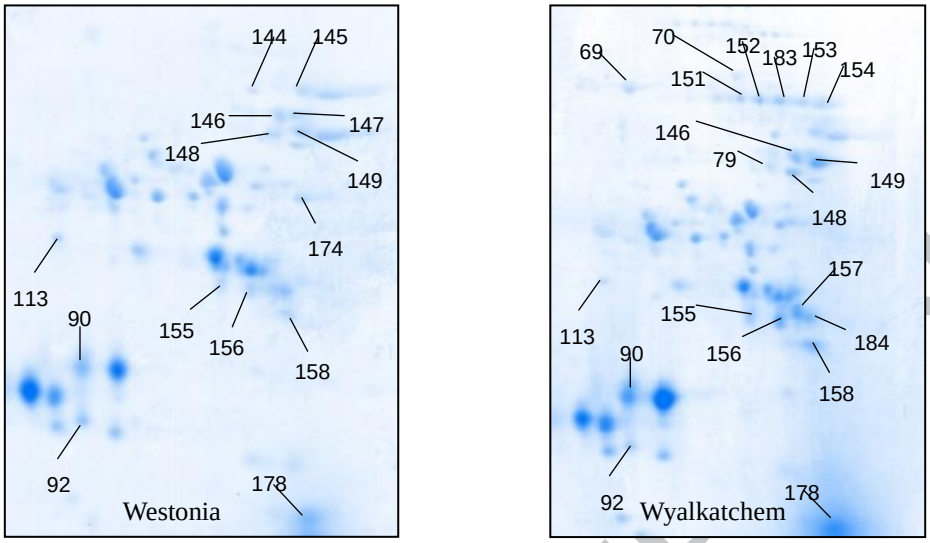


E: C3/32min (80 ℃)

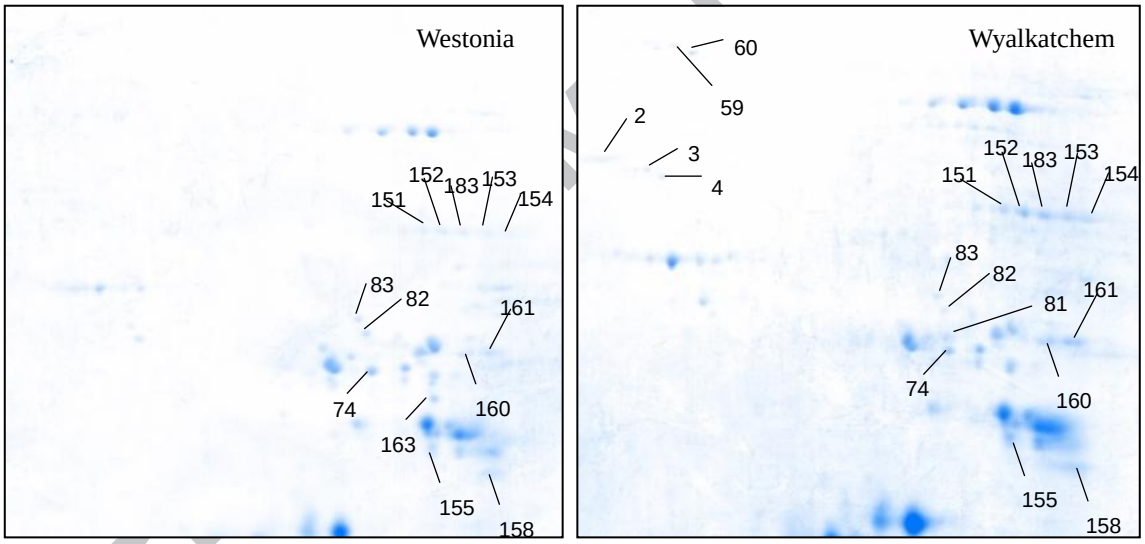




F: C4/38min (85 ℃)



G: 43min (80 ℃)



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- Mixolab proteomics expand our knowledge of the range of proteins in gluten matrix.
- Dynamic exchanges of gliadins and globulins in the gluten matrix are identified.
- Starch gelatinization stabilizes specific protein behaviour in the gluten complex.
- Varietal variation of protein behaviour confers varied flour quality attributes.
- The varied protein encoding genes are located to different chromosomes groups.